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Study Protocol for a Randomized Controlled Trial: Evaluating the Impact of Acupuncture on Menstrual Regulation and Pregnancy Enhancement in Patients with DOR Using Rs-fMRI to Assess Brain Functional Networks

Tianyu Bai*, E Zhou*, Kai Wang, Weizheng Li, Jieyu Bi, Jiacheng Ju

Department of Acupuncture and Moxibustion, Shandong Provincial Third Hospital Shandong University, Jinan, Shandong, 250031, People's Republic of China

*These authors contributed equally to this work

Correspondence: Tianyu Bai, Email Baitianyu19820217@163.com

Background: Resting-state functional magnetic resonance imaging (rs-fMRI) is a noninvasive way to evaluate brain physiological activity by detecting blood oxygen level fluctuations. Diminished ovarian reserve (DOR) indicates ovarian aging. Before 40, patients may have menstrual abnormalities, poor reproduction, and poor assisted reproductive results. Without treatment, it can cause early ovarian failure. Studies have shown that acupuncture can ameliorate sex hormones and antral follicle count (AFC) in DOR patients.
Objective: Despite limited studies on its mechanism, acupuncture have been shown to treat DOR. There is no relevant research on brain functional magnetic resonance and brain functional connectivity of acupuncture in treating DOR. We design this clinical trial to preliminarily elucidate the neuroimaging method of controlling the brain functional network and acupuncture impact in DOR patients using rs-fMRI.
Methods: This study will involve 30 DOR patients and 30 healthy individuals. DOR patients will have rs-fMRI before and after 3 menstrual cycles of acupuncture, whereas healthy individuals will need one rs-fMRI scan. The primary end measures will be follicle-stimulating hormone (FSH) and AFC. In contrast, the secondary outcomes will be luteinizing hormone(LH), estradiol (E2), anti-Müllerian hormone (AMH), modified Kupperman scale, self-rating anxiety scale (SAS), self-rating depression scale (SDS), and rs-fMRI alterations.
Results: This study uses rs-fMRI technology to identify the brain regions that differ between DOR patients and healthy people before and after acupuncture treatment. This study will connect brain regions, examine the effects of acupuncture on menstruation and pregnancy on DOR patients' brain function networks, and discuss neuroimaging methods.
Conclusion: Acupuncture may have the potential to regulate menstruation and increase the chances of pregnancy promotion in patients with DOR.
Keywords: clinical trials, study protocol, rs-fMRI, acupuncture, DOR, brain functional networks

Introduction

DOR is characterized by a decline in the quantity and/or quality of oocytes in the ovaries in women. This decline is accompanied by an elevation in FSH levels,[1] a reduction in AFC, and a decrease in AMH levels. Patients with DOR often exhibit decreased fertility, including infertility or difficulty in conception, early or recurrent miscarriage, poor ovarian response to ovulation-promoting drugs during assisted reproduction, or repeated embryo implantation failures.

The majority of individuals may experience regular menstruation, while it can also present as many menstrual disorders, encompassing alterations in the menstrual cycle, as well as irregularities, durations, and volumes.[2] The incidence rate of DOR is also increasing with the increase in life pressure and the change in the social environment.

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The American Association for Assisted Reproductive Technology (SART) registration shows that the prevalence of DOR has increased by 7% over 7 years.[3] DOR is considered the early stage of early-onset ovarian dysfunction (POI) or premature ovarian failure (POF). Studies have demonstrated that DOR can advance to POF within a timeframe of 1–6 years,[4] and prompt intervention has the potential to postpone or even reverse the development of POF.

The pathogenesis of DOR is unknown and is related to many factors such as genetics, autoimmune disease, infection, and environment.[5] There is no definite treatment measure for restoring ovarian function in clinical practice.[6] Western medicine treatment mainly focuses on hormone supplement treatment, ovulation promotion and assisted reproductive technology, but it cannot effectively restore ovarian function, and the long-term effect is poor, and long-term use of hormone increases the risk of thrombosis, breast cancer, endometrial cancer.[7] Therefore, finding a safe and effective treatment plan has become a major clinical challenge that urgently needs to be solved.

"Acupuncture for regulating menstruation and promoting pregnancy" is a highly effective acupuncture prescription that our team has long applied to the clinical treatment of DOR. The prescription takes tonifying the kidney essence, regulating Chongren, and tranquilizing the mind as the treatment principles, and it is widely used in clinical practice. A prospective case sequence study[8] showed that the acupuncture method of regulating menstruation and promoting pregnancy can reduce FSH levels and FSH/LH values and increase E2 levels and AFC in DOR patients. Professor Wang Xiao's research has found that the acupuncture method of regulating menstruation and promoting pregnancy can intervene in the hypothalamus, pituitary, hormone levels, ovarian tissue morphology, etc. of DOR patients, proving the exact efficacy of acupuncture treatment for DOR.[9]

Rs-fMRI reflects spontaneous neural activity by detecting the fluctuation of blood oxygen level in resting state and is a non-invasive method to study brain physiological activity.[10] Recently, scholars have applied the brain functional connectivity analysis method based on rs-fMRI technology to acupuncture research, showing that the regulation of acupuncture on the brain functional connectivity network is closely related to its effect mechanism. By analyzing the rs-fMRI signals, a large amount of information can be obtained on the functional connections, network connections, or organizational patterns of the resting brain regions, making it of significant research and clinical application value.[11]

In a prior study conducted by our research team,[12] it was shown that applying electroacupuncture to bilateral uterine acupoints activated various brain regions, including the anterior cuneiform lobe, cerebellum, posterior central gyrus, talus sulcus, and lingual gyrus. The potential correlation between neural activity in these specific brain regions and reproductive hormone levels, emotional fluctuations, somatosensory perception, and visual information has been suggested.[12]

Acupuncture has been proven to be clinically effective in treating DOR, but there are limited studies on the underlying process. The utilization of brain functional magnetic resonance (fMRI) technology in the investigation of acupuncture mechanisms is progressively growing. Particularly, examining brain functional connectivity and the effects of acupuncture has garnered increasing interest among researchers. However, currently, there is a dearth of pertinent research on the application of brain-functional magnetic resonance and brain-functional connectivity in the treatment of DOR through acupuncture.

Hence, the primary objective of this clinical research was to provide an initial understanding of the neuroimaging mechanisms involved in the regulation of the brain functional network and the effects of acupuncture in patients with DOR through the utilization of rs-fMRI technology.

Methods and Analysis

We will recruit 30 DOR patients and 30 healthy female volunteers of childbearing age from Shandong Provincial Third Hospital. The research plan has been approved by the Medical Ethics Committee of Shandong Provincial Third Hospital before the start of the study. All participants in this trial will receive unified training on the trial content, and patients and healthy volunteers need to sign an informed consent form before enrollment.

Participants

We plan to recruit 30 DOR patients and 30 healthy female volunteers of childbearing age through WeChat, poster posting, and other means (Figure 1). We will have a dedicated staff member responsible for patient recruitment, who will

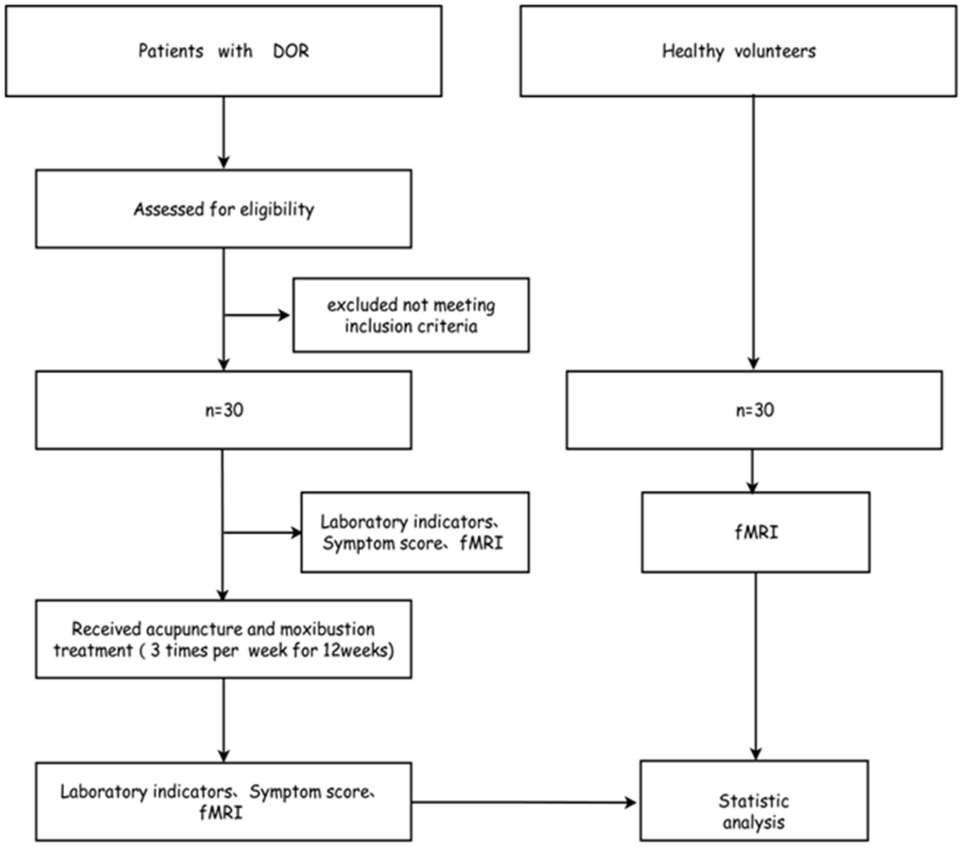

Figure 1 Trial flow chart.

record the patient's information in detail. The recruitment information will provide a detailed description of their contact information. This study complies with the Declaration of Helsinki.

Diagnostic Criteria

At present, there is no unified standard for the diagnosis of DOR. Referring to the "Chinese Obstetrics and Gynecology" edited by Cao Zeyi, the Bologna Conference standards of the European Association for Human Assisted Reproduction, the "Expert Consensus on Ovarian Hyporesponsiveness", and the "Expert Consensus on Clinical Diagnosis and Treatment of Ovarian Reserve Function Reduction", the following are proposed: (1) AMH<1.1ng/mL; (2) In the early follicular phase, vaginal ultrasound indicates that the number of follicles in bilateral ovarian sinuses (follicles with a diameter of 2–9mm) AFC is less than 5–7; (3) bFSH ≥10U/L for two consecutive menstrual cycles.

If any two of the above conditions are met, it can be diagnosed as DOR.

Inclusion Criteria

DOR Patients

(1) 18 years old ≤ age ≤ 40 years old;
(2) Right-handed;
(3) Meets DOR diagnostic criteria;
(4) The patient voluntarily accepts treatment and signs an informed consent form.

Healthy Volunteers

(1) 18 years old ≤ age ≤ 40 years old;
(2) Right-handed;

(3) Normal ovarian function;
(4) Normal immune function and no immune diseases;
(5) Informed and signed informed consent form.

Exclusion Criteria

DOR Patients

(1) Congenital genital dysplasia, bilateral ovariectomy patients;
(2) Patients with polycystic ovary syndrome, hyperprolactinemia, hyperandrogenism, hypothyroidism, chronic adrenal cortical dysfunction, and other endocrine diseases;
(3) Those with severe cardiovascular, cerebrovascular, liver, kidney, malignant tumors, hematopoietic system, and mental disorders;
(4) Patients who have received acupuncture treatment in the past month;
(5) Those with contraindications for magnetic resonance imaging, such as the installation of non detachable metal substances in the body, claustrophobia syndrome, etc.

Healthy Volunteers

Patients with severe cardiovascular, cerebrovascular, liver, kidney, malignant tumors, hematopoietic system, and mental disorders. Those with contraindications for magnetic resonance imaging, such as the installation of non detachable metal substances in the body, claustrophobia syndrome, etc.

Sample Size

The sample size calculation of the neuroimaging study is different from that of classic randomized controlled clinical trials. And because fs-MRI is expensive and does not allow for the large sample size that randomized controlled clinical trials do. It has been suggested that for fMRI studies, a minimum sample size ($n = 20$) should be used in order to obtain 80% power with an error threshold of 0.002 at a single-voxel level.[13] Considering a 20% dropout rate and to further enhance the reliability of the study results, the sample size of this study was increased to 30 per group, requiring a total of 60 participants.

Randomization and Blinding

The allocation order of the two groups will be generated by the computer. Due to the special nature of grouping, blind methods cannot be applied to DOR patients, healthy volunteers and acupuncture physicians, but blind methods are applied to laboratory physicians, outcome evaluation, data and functional magnetic resonance imaging processing personnel.

Interventions

DOR Group

Acupuncture was performed three times a week for 50 minutes each time, one menstrual cycle was a course of treatment, and three consecutive courses of treatment were performed.

Acupoint selection and acupuncture method: All acupoints will be located according to the World Health Organization Standardized Acupuncture Points Location.[14] Acupoint group 1:GV20 (Baihui), GV24 (Shenting), GB13 (Benshen), CV12(Zhongwan), ST25 (Tianshu), CV4 (Guanyuan), KI12(Dahe), ST29 (Guilai), ST36(Zusanli), SP6 (Sanyinjiao), KI3 (Taixi), LR3 (Taichong). Acupoint group 2: BL23(Shenshu) and BL32 (Ciliao) (Table 1). Operation: Use Huatuo brand disposable stainless steel filiform needles with specifications of 0.25*40mm and 0.30*75mm. CV12, ST25, CV4, ST29, KI12, SP6, LR3, ST36, all directly stabbed 20–30mm; KI3, and BL23, all piercing 5–20mm; GV20, GB13, GV24, stab 5–10mm back horizontally; BL32, with a downward oblique thrust of about 70 degrees, piercing 50–60mm into the posterior sacral foramen. The patient is placed in the supine position with acupuncture point group 1, and then in the prone position with acupuncture point group 2. All acupuncture points are required to receive qi and

Table 1 Acupoints Used in the Group 1 and Group 2

Group	Acupoints	Locations	Needle Insertion
Group 1	GV20(Baihui)	On the head, the front hair is 5 inches straight up from the center	Horizontally needle insertion at depth of 5–10mm
	GV24(Shenting)	On the head, the front hairline is 0.5 inches straight up from the center	Horizontally needle insertion at depth of 5–10mm
	GB13(Benshen)	On the head, 0.5 inches above the front hairline, and 3 inches beside the middle of the front	Horizontally needle insertion at depth of 5–10mm
	CV12(Zhongwan)	Upper abdomen, on the anterior midline, 4 inches above the navel	Perpendicular needle insertion at depth of 20–30mm
	ST25(Tianshu)	In the abdomen, horizontally flat navel, 2 inches apart from the anterior midline	Perpendicular needle insertion at depth of 20–30mm
	CV4(Guanyuan)	Located three inches blow the navel	Perpendicular needle insertion at depth of 20–30mm
	ST29(Guilai)	In the lower abdomen, 4 inches below the navel, 2 inches apart from the anterior midline	Perpendicular needle insertion at depth of 20–30mm
	KI12(Dahe)	In the lower abdomen, 4 inches below the navel, 0.5 inches apart from the anterior midline	Perpendicular needle insertion at depth of 20–30mm
	SP6(Sanyinjiao)	On the inner side of the calf, 3 inches above the inner ankle tip, behind the medial edge of the tibia	Perpendicular needle insertion at depth of 20–30mm
	KI3(Taixi)	In the depression between the inner ankle tip and the Achilles tendon in the ankle area	Perpendicular needle insertion at depth of 5–20mm
	LR3(Taichong)	In the anterior depression of the junction between the first and second metatarsals on the dorsum of the foot and metatarsal floor	Perpendicular needle insertion at depth of 20–30mm
	ST36(Zusanli)	On the outer side of the calf, 3 inches below the calf nose, 1 transverse finger outside the anterior tibial crest	Perpendicular needle insertion at depth of 20–30mm
Group 2	BL23(Shenshu)	In the spinal region, below the spinous process of the second lumbar vertebra, 1.5 inches apart from the posterior midline	Perpendicular needle insertion at depth of 5–20mm
	BL32(Ciliao)	In the sacrum, facing the 2nd posterior sacral foramen	Oblique needle insertion at depth of 50–60mm

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undergo the leveling, supplementing, and reducing method. Acupoint group 1 is left with acupuncture for 30 minutes, while point group 2 is left with acupuncture for 20 minutes.

Healthy Group

No treatment was given, only one functional magnetic resonance imaging scan was performed.

fMRI Scanning Program and Parameters

Using our hospital's Philips Ingenia 5.0T magnetic resonance scanner, data was collected using a 16 channel head and neck joint coil, with noise resistant earplugs inserted internally and sponge fixed externally.

Prior to the examination, the participants assume a supine position on the examination bed and engage in extended periods of rest in order to mitigate psychological influences, such as dread and anxiety. During the assessment, maintain a stationary head position, utilize eye masks, and instruct the individuals to unwind, refrain from falling asleep, and abstain from any cognitive tasks. The initial step involves scanning the subject's head using a positioning image. The scanning field of view for the head is then determined by considering the location of three anatomical sections: sagittal, coronal, and horizontal. Following the process of localization scanning, the initial step involves scanning T1 weighted structural pictures, which are subsequently followed by resting BOLD-fMRI sequence scanning.

3D-T1 Magnetic Resonance Imaging Data Scanning

Before completing the resting state fMRI scan, subjects need to first scan the 3D-T1 imaging data. The scanning parameters are: Repeat Time (TR)=6.008ms, Echo Time (TE)=1.70ms, and Field of View (FOV)=256 × 256mm^2, matrix=256 × 256mm^2.

BOLD-fMRI: Using Gradient Reduced Echo Planar Imaging (GRE-EPI) sequence to scan the resting state. Scanning parameters: TR=2000 ms, TE=30 ms, Inverse Angle (IA)=90°, number of layers = 31, layer thickness = 5mm, FOV = 240×240 mm^2, matrix = 64×64 mm^2.

Image Processing

Based on SPM12 and the Data Processing Assistant for Resting-State fMRI (DPARSF) software, fMRI data will be preprocessed on the Matlab R2015b platform, including removing the first 10 time points, time layer correction, head motion correction, spatial standardization, data smoothing, linear drift removal, and filtering.

Based on the functional connectivity (FC) analysis method of seed points, the common differences between DOR patients and healthy people and before and after acupuncture treatment in DOR patients will be taken as regions of interest (ROI), which were determined as seed points, the average BOLD signal time series of this ROI will be calculated, and then the correlation between all other regions and the average BOLD signal time series of this ROI will be calculated, The FC of the seed point can be obtained. Pearson correlation analysis will be used to analyze the average time series of seed points and the time series of various voxels in the whole brain, obtaining the correlation coefficient (r), which is the functional connectivity value between various sub points and other voxels in the whole brain. Fisher Z transformation was used to increase the normality of the data for statistical analysis.

Observed Indicators

Main Outcome Indicators

(1) Collect elbow vein blood from patients to measure FSH levels at early follicular stage before and three months after acupuncture treatment.

(2) Using transvaginal ultrasound, the number of 2–9mm follicles in both ovaries is measured during the early follicular phase to calculate the AFC. AFC measurements via transvaginal ultrasound are conducted before acupuncture treatment and again after three months of acupuncture treatment.

Secondary Outcome Indicators

(1) The values of LH and E2 will be measured by the electrochemiluminescence method. The elbow venous blood of DOR patients in the early follicular phase will be collected before and three months after acupuncture treatment.

(2) Blood samples were collected from the elbow vein of DOR patients during the early follicular phase for AMH testing, both before and after three months of acupuncture treatment.

(3) DOR patients' symptoms and emotional status before and after acupuncture intervention will be evaluated using the Modified Kupperman Scale, SAS, and SDS. The evaluation time points are before acupuncture treatment and three months after acupuncture treatment.

(4) Collect rs-fMRI data of DOR patients and healthy control groups before and three months after acupuncture treatment. Based on SPM12 and the Data Processing Assistant for Resting-State fMRI (DPARSF) software, fMRI data is preprocessed and analyzed on the Matlab R2015b platform.

Assessment of Safety

Adverse events will be obtained by acupuncture and therapists who ask participants after each treatment or by voluntary reports of participants.

Appropriate data on unfavorable incidents shall be documented for the whole experiment. Unbearable needle pricking pain, hematoma, broken or bent needles, and other adverse reaction events associated with acupuncture (symptoms like pain, nausea, vomiting, palpitations, dizziness, headache, anorexia, and insomnia that last for at least an hour after needle pricking).

Data Management and Monitoring

After the recording is completed, document management staff should rapidly gather the tabular documents. When documents are received, document management personnel should immediately check to ensure that they are correct, complete, and standardized. If there are any issues, they should be resolved as soon as possible. After validating the papers' correctness, completeness, and uniformity, document management professionals should put them in the appropriate folder and in a safe location. The data will be collected using paper questionnaires, kept totally confidential, and used only for the purposes of this study. The data will be kept for at least three years, and participants will be able to use their personal data in various ways, including checking, saving, maintaining, disclosing/not revealing, deleting, and/or processing or disposing of it.

Clinical Data Analysis

SPSS23.0 software will be utilized for statistical analysis of general data, enhanced Kupperman score, SAS score, SDS score, hormone three items, and AMH-related data. For quantitative data, the independent sample *t*-test (represented by $\overline{x} \pm S$) will be used. If normal distribution or homogeneity of variance is not fulfilled, the nonparametric rank sum test will be employed. For counting data, the Chi Square Test will be utilized, with results presented as a percentage. Pearson correlation analysis will be performed to investigate the relationship between various components, whereas Spearman correlation analysis will be utilized for data that does not fit to normality.

Discussion

The primary objective of this study is to provide an initial understanding of the efficacy and neuroimaging mechanisms associated with acupuncture therapy for DOR. While prior research[15–17] has established the positive impact of acupuncture on ovarian function and reproductive outcomes, there is a dearth of studies investigating the neuroimaging mechanisms underlying the effectiveness of acupuncture. Furthermore, the majority of high-quality research reports pertaining to acupuncture are currently limited to animal experimentation.

The rationale for choosing DOR patients as the focal group in this research proposal comes from the growing prevalence of delayed marriage and childbirth among women, coupled with the adaptation of China's fertility policies. Consequently, the proportion of elderly women requiring fertility assistance is expected to rise, leading to a substantial increase in the demand for fertility support among DOR women experiencing declining fertility rates.[5] Due to various factors such as environment, iatrology, and society, the fertility of young women is also greatly threatened. Early detection, diagnosis, and treatment of DOR are crucial in addressing this issue, as they play a vital role in formulating

suitable treatment strategies and reducing patients' time and financial burdens. Acupuncture has good effects both as a treatment measure for DOR and as a means of assisted reproductive pretreatment.[18]

We utilize FSH and AFC as the primary observation indicators to assess the efficacy of acupuncture. Previous studies have also confirmed that acupuncture can reduce FSH levels[19–21] and increase AFC counts.[19] Although AFC is somewhat subjective, having the same experienced senior physician perform the B-ultrasound examination can significantly minimize errors caused by subjectivity. AMH serves as an earlier and more sensitive biomarker for ovarian reserve, demonstrating strong predictive capabilities.[22,23] However, as it is not regulated by the hypothalamic-pituitary-gonadal axis, this indicator remains relatively stable,[24] and we will only utilize it as a secondary observation indicator. Some patients with DOR experience symptoms such as hot flashes, night sweats, fatigue, poor sleep, anxiety, and depression. Previous studies have indicated that acupuncture can alleviate these symptoms,[25] thus we choose the modified Kupperman, SAS, and SDS to assess the therapeutic effects of acupuncture. Naturally, mounting evidence suggests that acupuncture can enhance patients' sleep and mood by modulating the functional activities of emotion-related brain regions.[26–28]

Our team uses acupuncture as a powerful treatment for disorders causing ovarian function decline. This approach helps regulate menstruation and promote pregnancy. Its main meanings include whole cycle acupuncture therapy, paying attention to calming the mind, and controlling menstruation to encourage pregnancy. Strong promotion and application value, easy operation, good repeatability, and fewer acupoints are the features of this prescription.[29]

Based on rs-fMRI technology, this research scheme takes the common difference brain regions between DOR patients and healthy people and before and after acupuncture treatment of DOR patients as regions of interest, carries out brain functional connection with other brain regions, looks for the regulation of acupuncture for regulating menstruation and promoting pregnancy on the brain function network of DOR patients, and then discusses the neuroimaging mechanism of acupuncture treatment of DOR. This project is still blank in this field at present.

There are still several shortcomings in this research plan: firstly, the sample size is small, and the control group does not undergo any intervention, which may exaggerate the therapeutic effect of acupuncture; Secondly, the control group consists of healthy volunteers, and there is no established standard for the concept of "health", which cannot exclude the impact of other minor diseases on brain functional connectivity results, resulting in biased experimental results; Thirdly, there is currently no internationally recognized diagnostic standard for DOR. The diagnostic standard for this protocol is mainly based on the domestic Consensus of Clinical Experts on Diagnosis and Treatment of Ovarian Reserve Deficiency. Whether there are regional, ethnic, and lifestyle biases in this standard remains to be discussed.

Conclusion

Acupuncture may play a role in regulating and improving the ovarian function of DOR patients by adjusting the functional activities of certain brain regions. This study may provide imaging evidence for acupuncture treatment of diseases related to ovarian dysfunction.

Patient and Public Involvement

Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Ethics Approval

This study was approved by the Medical Ethics Committee of Shandong Provincial Third Hospital on March 21, 2023 (approval number: 2023016), and registered in Chinese Clinical Trial Registry (registration number: ChiCTR2300070142). The research results will be published in peer-reviewed journals.

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Disclosure

Tianyu Bai and E Zhou are co-first authors of the article. The authors report no conflicts of interest in this work.

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